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Citation

Cai, Xiao-xiao, En Luo, and Quan Yuan. 2010. "Interaction between Schwann Cells and Osteoblasts In Vitro." *International Journal of Oral Science* 2 (2): 74-81. doi:10.4248/IJOS10039. <http://dx.doi.org/10.4248/IJOS10039>.

Published Version

doi:10.4248/IJOS10039

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Interaction between Schwann Cells and Osteoblasts *In Vitro*

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Abstract

Aim Given the well-known properties of Schwann cells in promoting nerve regeneration, transplanting Schwann cells into implant sockets might be an effective method to promote sensory responses of osseointegrated implants. The aim of this study was to evaluate the interaction between Schwann cells and osteoblasts.

Methodology Schwann cells derived from the sciatic nerves of neonatal rat were co-cultured with osteoblasts using Transwell inserts. The proliferation of Schwann cells in the co-culture system was evaluated using methylthiazol tetrazolium (MTT) colorimetric method. Moreover, the secretions and mRNA levels of brain-derived neurotrophic factor (BDNF) and nerve growth factor (NGF) were measured by enzyme-linked immunosorbent assay (ELISA) and quantitative real-time PCR, respectively. In order to test the effect of Schwann cells on osteoblasts, alkaline phosphatase (ALP) staining and Alizerin red staining were

performed as well.

Results Schwann cells, which were co-cultured with the osteoblasts, showed an intact proliferation during the observation period. Moreover, the gene expression and synthesis of BDNF and NGF were not impaired by the osteoblasts. Meanwhile, co-cultured osteoblasts exhibited a significant increase in the proliferation on day 3 and 6 ($P < 0.05$). Co-culture of these two types of cells also led to a more intense staining of ALP and an elevated number of calcified nodules.

Conclusion These findings demonstrate that, in the *in vitro* indirect co-culture environment, Schwann cells can maintain their normal ability to synthesize neurotrophins, which then enhance the proliferation and differentiation of osteoblasts.

Keywords osteoblasts, Schwann cell, co-culture, proliferation, differentiation

Received Feb. 20, 2010; Revision accepted Mar. 23, 2010

Introduction

Osseointegrated dental implants have been widely used in clinics and regarded as useful and reliable treatment for the replacement of missing teeth. Studies on implant loading have shown, however, that the sensory thresholds of an implant are 10–100 times higher than those of natural teeth (Muhlbradt *et al.*, 1989; Jacobs and van Steenberghe, 1991; Hammerle *et al.*, 1995). Although some nerve fibers were observed in the vicinity of osseointegrated implants, and suggested to be the key elements contributing to sensory responses of external mechanical loading (Wada *et al.*, 2001; Ysander *et al.*, 2001), these nerve fibers were

present at quite a low density, resulting in a much higher sensory threshold than that of natural teeth (Muhlbradt *et al.*, 1989; Jacobs and van Steenberghe, 1991; Hammerle *et al.*, 1995). Without an efficient self-protective mechanism afforded by the periodontal ligament, excessive forces may be loaded on the implant, and may result in damage to peri-implant bone (Misch *et al.*, 2005). Therefore, improving the sensory responses of osseointegrated dental implants with a method involving use of a Schwann cell graft has been proposed (Yuan *et al.*, 2007).

Schwann cells are the glial cells of the peripheral nerve system, and play indispensable roles during neural development and regeneration (Fros-

tick *et al.*, 1998; Johnson *et al.*, 2005). In periodontal ligament, Schwann cells extend their cytoplasmic processes toward the axon terminals of Ruffini endings and wrap themselves around them. Some bioactive molecules produced by Schwann cells make an important contribution to the maturation and regeneration of periodontal Ruffini endings (Alkhamrah *et al.*, 2003; Wakisaka and Atsumi, 2003). When a Schwann cell graft has been applied to repair peripheral nerve injuries, it has been reported to have a preferential effect on functional recovery (Hess *et al.*, 2007; Wakao *et al.*, 2010). Therefore, a similar performance might be anticipated when Schwann cells are transplanted into implant sockets.

To verify this hypothesis, we previously studied cytocompatibility between Schwann cells and titanium implants. Schwann cells were seeded on various titanium implant surfaces and their morphology and functions were tested. The results demonstrated that Schwann cells could proliferate well on the titanium surface, and maintain their properties in nerve regeneration (Yuan *et al.*, 2010). Another prerequisite that must be clarified, however, is whether Schwann cells will interfere with the process of osseointegration around dental implants. As is known, osteoblasts are mainly responsible for the new bone formation around dental implants during the initial process of osseointegration. We therefore studied the effect of Schwann cells on osteoblasts, and found that the proliferation and differentiation of osteoblasts could be induced, suggesting Schwann cells transplantation should not affect the process of osseointegration (Yuan *et al.*, 2008). We have been unable to identify, however, any report examining the effect of osteoblasts on the biological functions of Schwann cells. The purpose of the present study was to further investigate the interaction between these two kinds of cells using a novel *in vitro* co-culture system.

Material and Methods

Primary culture of Schwann cells

Primary Schwann cells were obtained by methods similar to those first described by Brookes *et al.*

(Brookes *et al.*, 1979). Briefly, the bilateral sciatic nerves of neonatal Sprague-Dawley rat pups (day 2) were dissected and washed three times with phosphate-buffered saline (PBS, pH 7.4). The epineurium of each nerve was stripped under the stereomicroscope. Nerves were cut into pieces (<1 mm) and enzymatically dissociated using 0.1% type I collagenase (Sigma, USA) for 30 minutes and 0.25% trypsin (Sigma, USA) for 10 minutes at 37°C. The resulting cell suspension was centrifuged (1 000 r·min⁻¹, 8 min), placed into 25 mm² culture flasks (Costar Corning, USA) and incubated in Dulbecco's Modified Eagle's Medium (DMEM, Gibco, USA) supplemented with 10% fetal bovine serum (FBS, Gibco, USA) and antibiotics (penicillin-streptomycin solution). The antimetabolic agent cytosine arabinoside (10⁻⁵ mol·L⁻¹) was added to the culture medium 24 hours after initial plating and incubated for 2–3 days to remove fibroblasts. Finally, Schwann cells were confirmed with the S-100 protein staining technique. The number of S-100 positive and negative cells were counted at 20 randomly selected fields under a microscope to calculate the purity of the cultured Schwann cells, which was found to be more than 93%.

Primary culture of calvarial osteoblasts

Osteoblasts enriched cells were enzymatically isolated from calvariae of the same rat pups where Schwann cells had been harvested by the method described previously (Hinoi *et al.*, 2001). Briefly, dissected calvariae were sequentially digested for 10 minutes intervals with 0.1% type I collagenase (Sigma, USA) and 0.25% trypsin (Sigma, USA) at 37°C. Cells obtained from the second to the fourth digestions were incubated in DMEM (Gibco, USA) with antibiotics and 10% FBS (Gibco, USA). At about 90% confluence, cells were trypsinized and subcultured. To confirm that cultures were osteoblast enriched, mineralized bone nodule formation assay and alkaline phosphatase (ALP) staining were performed (data not shown). All experiments were performed using the cells of third or fourth passage.

Co-culture of Schwann cells and osteoblasts

The co-culture system was established using

six-well Transwell clear polyester membrane inserts with 0.4 µm pore size (Costar Corning, USA). Osteoblasts and Schwann cells were seeded in the tissue culture plates and Transwell inserts, respectively, at the ratio of 1 : 1 (Group A) or 2 : 1 (Group B). After one day, Schwann cells inserts were transferred into the wells containing osteoblasts, so that two different cell types shared the culture medium but were not in physical contact. Nutrient media (DMEM with 10% FBS and antibiotics) were changed every three days.

Assessment of cell proliferation

Cell proliferation was assessed using a methylthiazol tetrazolium (MTT, Sigma, USA) method. Briefly, 1 mL serum free medium and 100 µL MTT solution (5 mg·mL⁻¹) were added to each sample, followed by incubation for 4 hours at 37°C for MTT formazan formation. The medium and MTT were then replaced by 1 mL dimethyl sulphoxide (DMSO) to dissolve the formazan crystals. After 30 minutes, 200 µL of supernatant was transferred to microplate wells (Falcon), and the optical density was quantified against a DMSO solution blank in a microplate reader at the wavelength of 492 nm (HTS 7000 plus, Perkin-Elmer, USA).

Enzyme-linked immunosorbent assay

To quantify the concentration of brain-derived neurotrophic factor (BDNF) and nerve growth factor (NGF) in cell cultured supernatant, commercially available enzyme-linked immunosorbent assay (ELISA) kits were used strictly according to the manufacturer's instruction (Promega, USA). Prior to analysis, the supernatant was centrifuged to

remove cell debris.

RNA extraction and quantitative real-time RT-PCR

Total RNA was extracted from each sample by the TRIZOL method strictly following the manufacturer's protocol (Invitrogen, Carlsbad, USA). The concentration and purity of freshly isolated RNA were measured at 260 nm using a spectrophotometer and with $A_{260/280}$ ratio respectively. The first-strand cDNA was synthesized from 1 µg RNA with murine leukemia virusreverse transcriptase (Takara, Japan), and used for quantitative real-time PCR. Expression levels of BDNF and NGF were quantified with an ABI 7300 real-time PCR system (Applied Biosystems, USA) and SYBR green PCR reaction mix (Infinigen, USA). Primers designed in primer express 2.0 analysis software were listed in Table 1. The program used was 50°C for 2 minutes, 95°C for 10 minutes, 40 cycles of 95°C for 15 seconds and 60°C for 1 minute. Melting analysis and agarose gel electrophoresis were performed to confirm the specificity of the PCR products obtained using each primer pair. The relative expression levels of genes were analyzed using the $2^{-\Delta\Delta C_t}$ method by normalizing with β -actin house-keeping gene expression, and presented in fold increase relative to the control group.

ALP staining

To test the differentiation of osteoblasts, after nine days of co-culture, osteoblasts were washed with PBS and stained with the commercial kit according to the manufacturer's instruction (Sigma, USA).

Table 1 Specific primers designed following the cDNA sequences of each gene in GenBank

Gene	Genbank accession number	Primers	Product size
BDNF	NM_012513	(+), 5'-GGAGGCTAAGTGGAGCTGACATAC-3'	225 bp
		(-), 5'-GTGCTTCCGAGCCTTCCTTAGG-3'	
NGF	XM_001067130	(+), 5'-CCAAGCACTGGAACCTACTACTGC-3'	142 bp
		(-), 5'-CTGCTGAGCACACACGCAG-3'	
β -Actin	NM_031144	(+), 5'-CACGGCATTGTCACCAACTG-3'	167 bp
		(-), 5'-TTGAAGGTCTCAAACATGATCTGG-3'	

Alizarin red staining

The ability of osteoblasts to form calcified bone nodules was assessed using Alizarin red staining technique. After co-culture with Schwann cells for 21 days, osteoblasts were washed, fixed in 4% formaldehyde, incubated for 10 minutes at room temperature in 1% Alizarin red solution (pH 4.2), and then rinsed with PBS.

Statistical analysis

All experiments presented here were run three times with triplicate samples. Data were shown as means \pm SD, and statistically analyzed using one-way ANOVA followed by Newman-Keuls post hoc tests. Differences with a probability of occurring by chance alone of less than 0.05 were considered statistically significant.

Results

Intact proliferation of Schwann cells

To test the proliferation of Schwann cells in the co-culture system, we performed MTT assay on day 3, 6, and 9. During the observation period, the OD values of each group increased steadily (Figure 1), and no significant difference was noticed among

the three groups, suggesting the proliferation of Schwann cells would not be affected by the co-cultured osteoblasts.

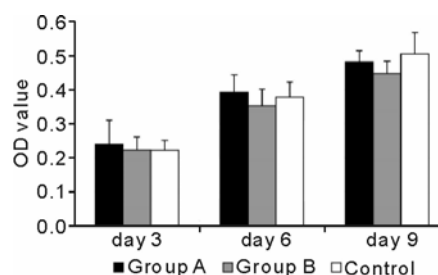


Figure 1 Proliferation of Schwann cells co-cultured with the osteoblasts

The presence of osteoblasts did not impair the proliferation of Schwann cells.

Unimpaired synthesis and gene expression of BDNF and NGF in Schwann cells

The concentrations of BDNF and NGF in the culture medium were measured on days 3, 6 and 9, and no significant difference was observed during the whole observation period (Figure 2), suggesting Schwann cells could maintain their functions of secreting neurotrophic factors even in the presence of osteoblasts. To further analyze the gene expression of these two factors, real-time quantitative PCR was performed, and it was noted that the mRNA levels of BDNF and NGF were elevated on day 6 and day 3, respectively (Figure 2).

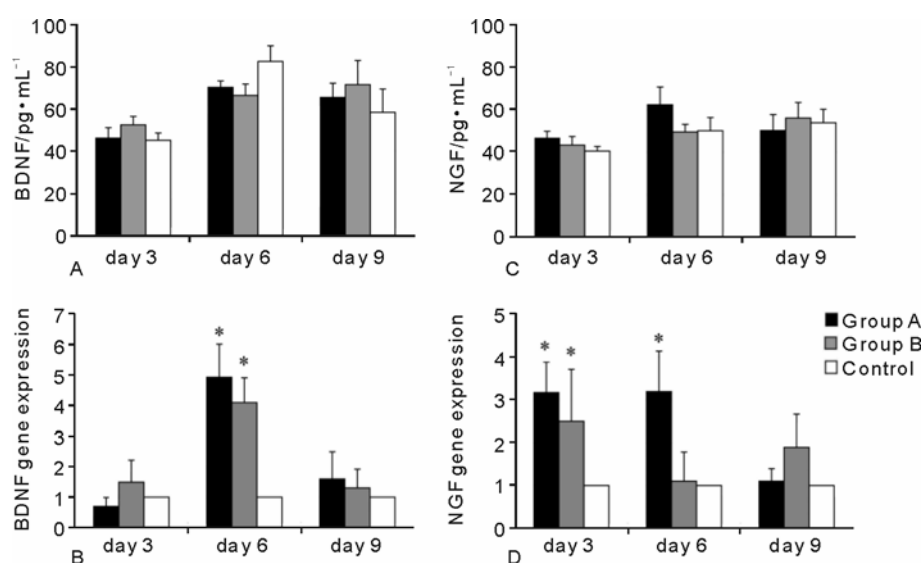


Figure 2 The effect of co-culture on synthesis and gene expression of BDNF and NGF in Schwann cells

There is no significant difference on the secretion of BDNF and NGF during the whole observation period (A and B), although the mRNA levels of BDNF and NGF were elevated on day 6 and day 3, respectively (C and D). * $P < 0.05$.

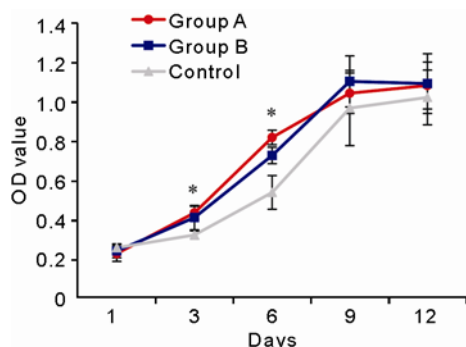


Figure 3 Proliferation of co-cultured osteoblasts measured by MTT assay

On day 3 and 6, both Group A and B had significantly higher levels of osteoblast viability than that of the control.

Increased the proliferation of osteoblasts

We further asked whether neurotrophic factors secreted by Schwann cells are biologically active. As shown in Figure 3, co-culture of Schwann cells stimulated the proliferation of rat primary osteoblasts as measured by MTT assay. On day 3 and 6, both Group A and Group B had significantly higher levels of osteoblast viability than that of the control ($P < 0.05$, see Figure 3). After nine days of co-culture, cells of all three groups reached high confluence, and no significant difference was observed.

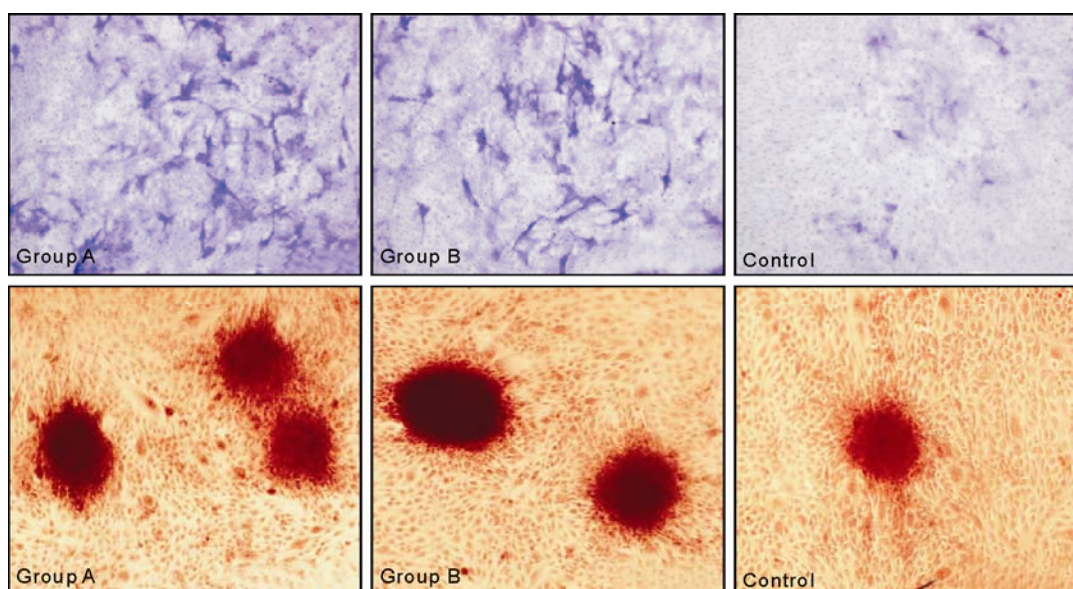


Figure 4 ALP staining (upper panel) and Alizarin red staining (lower panel) of osteoblasts

Compared with the control, co-cultured osteoblasts exhibited more intense ALP staining and many more mineralized bone nodules.

Schwann cells enhance the differentiation and mineralization of osteoblasts

To further evaluate the effect of neurotrophic factors on differentiation of osteoblasts, we performed ALP staining after nine days of co-culture. As shown in Figure 4, both Group A and Group B exhibited much more intense ALP staining, suggesting Schwann cells could induce the ALP activity of osteoblasts in the indirect co-culture condition. To analyze whether Schwann cells could improve the mineralization of osteoblasts, we stained the osteoblasts with Alizarin red on day 21. As shown in Figure 4, the mineralized bone nodules

were visualized by as red-purple spots. Compared with the control, both co-cultured groups had an increased number and size of calcified nodules, with more intense red staining.

Discussion

In this study, our results showed some upregulation of BDNF and NGF gene, but unimpaired secretion of BDNF and NGF peptides, which suggest that Schwann cells can maintain their functions of secreting neurotrophic factors even in the presence of osteoblasts, and these factors may

enhance the process of osseointegration and facilitate the regeneration of nerve endings in the peri-implant region.

It is well-known that a variety of neurotrophic factors, NGF, BDNF and neurotrophin-3 (NT-3), are synthesized and released by Schwann cells (Frostick *et al.*, 1998; Johnson *et al.*, 2005; Bhatheja and Field, 2006). Among them, BDNF and NGF are two key neurotrophins (Frostick *et al.*, 1998; Johnson *et al.*, 2005; Bhatheja and Field, 2006). BDNF initiates its biological effect through binding to specific transmembrane tyrosine kinase receptor (trkB) (Bothwell, 1995), and plays an important neuroprotective role in nerve system. Alkhamrah *et al.* reported that the null mutant of BDNF induced a malformation and reduction of Ruffini endings in periodontal ligament (Alkhamrah *et al.*, 2003), suggesting BDNF is required in the development and maturation of periodontal Ruffini endings. NGF is the first and best-characterized member of the neurotrophins (Wiesmann and de Vos, 2001). It plays a crucial role in regulating differentiation and survival of neurons in central and peripheral nerve systems. External NGF can enhance the regeneration of not only neural tissues, but also non-neural tissues, such as ligament, bone and periodontal membrane (Wang *et al.*, 2006; Xu *et al.*, 2006; Mammoto *et al.*, 2008).

Although our data demonstrated that Schwann cells could maintain their functions of secreting neurotrophic factors in the presence of osteoblasts, whether these secreted neurotrophins are biologically active are still unknown. We, therefore, evaluated their effect on the proliferation and differentiation of co-cultured osteoblasts. Consistent with our previous report (Yuan *et al.*, 2008), osteoblasts co-cultured with Schwann cells proliferated more rapidly than osteoblasts cultured alone. Furthermore, co-cultured osteoblasts exhibited more intense staining of ALP and an elevated number of calcified nodules. These results indicated that the neurotrophic factors secreted by Schwann cells are biologically active, and could enhance the proliferation and differentiation of osteoblasts.

Some studies showed that the neurotrophins secreted by Schwann cells may bind to their receptors located on cell membrane of not only sympathetic and sensory neurons, but also various

types of non-neuronal cells, including osteoblasts epithelial cells, dental pulp cells, tumor cells, and so on (Mizuno *et al.*, 2007; Lee *et al.*, 2008; Sigala *et al.*, 2008; Truzzi *et al.*, 2008). The presence of trkA, trkB and trkC, which are the receptors of NGF, BDNF, and NT-3 respectively, has also been reported in osteoblastic cells (Nakanishi *et al.*, 1994a; Nakanishi *et al.*, 1994b; Asaumi *et al.*, 2000). NGF was suggested to play an important role in the survival of osteoblastic cells (Mogi *et al.*, 2000). In addition, Yada *et al.* found that NGF might promote ALP activity and collagen biosynthesis of osteoblastic MC3T3-E1 cells (Yada *et al.*, 1994). Recently, outstanding stimulation of proliferation, ALP and COL-1 mRNA expression by NGF in human pulp cells was also reported (Mizuno *et al.*, 2007). Our previous data also demonstrated that the presence of Schwann cells could significantly induce the expression of osteogenic marker genes, including ALP, OCN and COL-1 (Yuan *et al.*, 2008). More importantly, in this study, we observed a much more intense staining of ALP and increased number of calcified nodules. Taken together, these findings indicate that Schwann cells enhance the differentiation of primary osteoblasts through a NGF pathway.

Conclusion

The findings of this study suggest that Schwann cells can maintain their own functions of secreting neurotrophic factors even in the presence of osteoblasts. The secreted factors are biologically active as they could improve the proliferation, differentiation and mineralization of osteoblasts. Further experiments are needed to exclude the limitation of the *in vitro* culture system used in this study.

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